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Variations of non-protein nitrogen in six Spanish legumes according to the extraction method used

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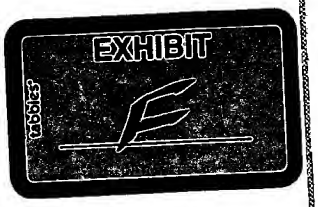

The non-protein nitrogen content (NPN) of six edible grain legumes (pea, lentil, faba bean, chick pea, field bean and lupin) grown in Spain were evaluated. NPN was extracted by three methods (70% ethanol, 70% ethanol + 1% TCA and 0.2% NaOH + 30% TCA) and then analyzed using the micro-Kjeldahl method. The extraction methods including TCA provided higher NPN values than the method which only used 70% ethanol. High concentrations of glutamic and aspartic acids and low concentrations of methionine and cystine were found in the NPN. However, the total NPN fraction and the free amino acid profiles obtained by each extraction method differed as a consequence of the differing solubility of the α-amino groups and other components of the NPN fraction. Further studies should be conducted to obtain a suitable extraction method for measuring total NPN in legumes. Copyright © 1996 Canadian Institute of Food Science and Technology. Published by Elsevier Science Ltd

Keywords: non-protein nitrogen, legumes, protein nitrogen, total nitrogen.

INTRODUCTION

Food legumes contain two to three times the protein content of cereals and provide variety and a flavorful complement to bland cereal-based diets in which meat is in short supply (Phillips, 1993). In addition, they are widely accepted in most developing countries since they are not forbidden by any religious code and, based on their low cost, they also provide the best means of combatting malnutrition (Savage, 1988)

The total protein contents of food legumes vary from 20 to 35% (expressed on a dry weight basis), depending on the kind of legume seed, cultivar and environmental growing conditions (Geervanni & Devi, 1988). In the normal procedure for estimating protein intake, the nitrogen content is calculated by the standard micro-Kjeldahl method and a factor is used to convert the figure into protein percentage. In this process, it is tacitly assumed that all the nitrogen is associated with the protein although, in fact, this is not strictly true (Mossé, 1990). For this reason, any large variation in the non-protein nitrogen (NPN) content would affect the estimated protein intake in the diet, particularly in legumes, in which the protein content is thought to be overestimated by at least 2–4% on a seed weight basis (Desphande & Nielsen, 1987). However, some legume NPN consists of nucleic acid nitrogen and α-amino acid nitrogen, which might be of important nutritional value (Singh & Jambunathan, 1981; Kochhar & Walker, 1988), as has been found in other foods such as seafood (Shahidi *et al.*, 1993), milk (Allegri *et al.*, 1993) and mushrooms (Fujihara *et al.*, 1995).

The research presented here was an attempt to evaluate the NPN content of six grain legumes commonly cultivated in the Spanish Mediterranean area, using three extraction methods, as a contribution to our knowledge of the Mediterranean diet.

MATERIALS AND METHODS

Six grain legumes produced in the Spanish Mediterranean area were evaluated in the present study. Samples were purchased from local markets in the following commercial appearance: lentils (*Lens culinaris*), faba beans (*Vicia faba*), chick peas (*Cicer arietinum*) and field

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beans (*Phaseolus vulgaris*) as dry seeds, and green peas (*Pisum sativum*) and lupins (*Lupinus luteus*) as fresh seeds, which were frozen and dried for 36 h in a Virtis freeze-dryer model 10-324 (Gardiner, NY, USA). All dried seeds were ground to a powder in a coffee mill and then passed through a US standard 25-mesh sieve. The meal samples were placed in a drier at 25°C overnight to obtain uniform moisture of over 5% in all samples.

Assays were carried out in triplicate for all analyses and the results expressed on a dry weight basis. In all the analytical methods, mean ± standard deviation (SD) values were calculated.

Total and protein nitrogen

Legume meals were assayed for total N by the micro-Kjeldahl method (AOAC, 1990). Protein N was determined by the trichloroacetic acid (TCA) precipitation method (Awolumate, 1983). One hundred mg of meal was solubilized in 5 ml of a 0.2% NaOH (w/v) solution, shaken for 20 min, and centrifuged at 6000×*g* for 5 min at room temperature. The precipitate was washed with 2 ml of the 0.2% NaOH and added to the supernatant after a second centrifugation under the same conditions, and TCA added (5 ml). The samples were left for 2 h at 4°C with occasional shaking and then centrifuged at 12000×*g* for 20 min at room temperature. The precipitate was washed with 5 ml of ethyl ether, dried for 30 min at 105°C and weighed. The nitrogen content of the precipitate was then determined by the micro-Kjeldahl method.

Non-protein nitrogen

The NPN was estimated as the difference between total N (micro-Kjeldahl method) and protein N (Awolumate method), obtaining the fraction called TN PN. The values of this fraction (TN–PN) were then compared with the NPN values obtained using three extraction methods. NPN extraction of the six grain legumes was based on Bhatty's procedure (Bhatty *et al.*, 1973). Three extraction methods were used to determine any variability in the amount of nitrogen extracted.

Ethanol method (EtOH)
One gram of meal was shaken for 1 h at room temperature (22°C) with 70% (v/v) ethanol (EtOH) in a meal/solvent ratio of 1:20 w/v. The insoluble materials were removed by centrifugation (10000×*g*; 10 min) at room temperature.

Ethanol and TCA method (EtOH + TCA)
This method was the same as the EtOH method, except that the solvent was 70% (v/v) EtOH + 1% (w/v) trichloroacetic acid (TCA), which was added to improve nitrogen extraction.

Sodium hydroxide and TCA method (NaOH + TCA)
Three grams of each meal were extracted with 80 ml of 0.2% NaOH (w/v) (pH 12) for 90 min in a shaker. The insoluble material was removed by centrifugation (10000×*g*; 10 min) at room temperature, and 80 ml of 30% TCA (w/v) was added to the supernatant. The mixture was stirred for 5 min at room temperature and the protein removed by centrifugation (10000×*g*). In this case, the alkali-soluble protein was first extracted with 0.2% NaOH solution and then precipitated with the 30% TCA, and the NPN was solubilized in the supernatant.

The micro-Kjeldahl standard method (AOAC, 1990) was used to analyzed the total N content in an aliquot of each supernatant obtained using the above extraction methods. Attempts were also made to calculate the amount of solubilized protein N in each extraction by using the Lowry procedure (Lowry *et al.*, 1951). The true NPN of each grain legume was calculated as the difference between the N as determined by micro-Kjeldahl method and the solubilized protein N of the supernatants as determined by Lowry *et al.* (1951).

Free amino acids

For free amino acid analysis, the supernatant fraction of NPN extracted with each method was filtered through a Millipore filter (type GV; pore size, 0.22 μm, ref. no. GVWP01300) and an aliquot was analyzed in an amino acid analyzer LKB Alpha Plus (Pharmacia LKB Biochrom Ltd., Cambridge, England).

Amide nitrogen

The amide N solubilized with each extraction method was calculated by reference to the molecular weight of the nitrogen content in free amino acids.

RESULTS AND DISCUSSION

Data concerning the total and protein N, and TN–PN contents of the six different Spanish legumes investigated are summarized in Table 1. The total N content of whole seeds ranged from 3650 to 5820 mg/100 g for chick pea and lupin, respectively. These values, determined by the micro-Kjeldahl method, might give a misleading idea of the actual protein content, as can be observed from the adjacent column, which shows the protein N as determined by the TCA precipitation method of Awolumate (1983). Using this method, protein N ranged from 1700 mg/100 g for pea seed to 4550 mg/100 g for lupin seed, which would represent more than 50% of the total N content of the grain legumes, except in the case of pea seed, which showed the lowest percentage of protein N (only 38.46% of the total N). Assuming that the micro-Kjeldahl method is

Table 1. Total nitrogen, protein nitrogen and non-protein nitrogen (NPN = Total N–Protein N) of grain legumes[a]

Legume	Total N	Protein N		NPN	
	mg/100 g	mg/100 g	% of Total N	mg/100 g	% of Total N
Chick pea	3650 ± 50	2390 ± 20	65.21	1270 ± 60	34.79
Faba bean	3920 ± 40	2900 ± 70	73.98	1020 ± 70	26.02
Field bean	4050 ± 130	2700 ± 20	66.67	1350 ± 23	33.33
Lentil	4270 ± 110	2505 ± 10	59.72	1720 ± 70	40.28
Lupin	5820 ± 30	4550 ± 38	78.18	1270 ± 33	21.82
Pea	4420 ± 80	1700 ± 35	38.46	2720 ± 10	61.54

[a]Results are mean values ± SD of three replicates. Data are expressed on a dry weight basis.

Table 2. Non-protein nitrogen (NPN) and amide nitrogen contents in six legumes using three extraction methods (mg/100 g on a dry weight basis)[a]

Legume	EtOH		EtOH + TCA		NaOH + TCA	
	NPN	Amide N	NPN	Amide N	NPN	Amide N
Chick pea	107 ± 3	79 ± 2	713 ± 8	156 ± 4	520 ± 8	264 ± 3
Faba bean	239 ± 12	138 ± 15	831 ± 15	102 ± 7	282 ± 6	225 ± 2
Field bean	203 ± 13	148 ± 4	425 ± 9	167 ± 2	523 ± 8	376 ± 1
Lentil	270 ± 7	203 ± 3	518 ± 17	486 ± 5	1049 ± 17	181 ± 5
Lupin	110 ± 6	55 ± 3	169 ± 7	121 ± 3	480 ± 25	150 ± 4
Pea	924 ± 23	19 ± 4	1155 ± 36	49 ± 7	1200 ± 17	975 ± 8

[a]Results are mean values ± SD of three replicates.

an accurate and suitable technique for determining the total N content of a food, and Awolumate's method is suitable for estimating the protein N content, TN PN could give an estimated value of the content of NPN. The TN–PN values ranged from 1020 to 2720 mg/100 g for faba bean and pea, respectively. It is clear that the NPN content represents a substantial proportion of the total N content of legumes, meaning that there is an overestimation of protein intake in the diet when protein content of legumes is calculated using the micro-Kjeldahl method, as has been shown by other authors (Sosulski & Holt, 1980; Mossé, 1990; Wu *et al.*, 1995).

To better understand the true NPN content of these seeds, the effect of three extraction methods was tested using different solvents to solubilize the NPN: EtOH, EtOH + TCA, and NaOH + TCA. Table 2 shows the NPN and amide nitrogen contents solubilized with the three extraction methods in the six grain legumes studied. All values for NPN were corrected by reference to the protein N content solubilized with each extraction method, as determined by Lowry's colorimetric technique (Table 3).

In general, the NPN content depends on the cultivar (Singh *et al.*, 1981*a*; Singh & Jambunathan, 1981; Drumm *et al.*, 1990) and on seed maturity (Ros & Rincón, 1990, 1993). Furthermore, there are substantial variations between the NPN content of legumes and oilseeds depending on the extraction method used (Bhatty *et al.*, 1973; Singh & Jambunathan, 1981). In our study clear differences can be observed between the NPN and amide N solubilized by each method. The EtOH + TCA and NaOH + TCA methods gave more NPN than did the method using EtOH alone. The first method solubilized more NPN in chick pea and faba bean, whereas NaOH + TCA gave higher values for NPN solubilized in field bean, lentil, lupin and pea. There were substantial differences in the TCA-soluble N content (EtOH + TCA and NaOH + TCA methods) of the six legumes, which were in general agreement with the data reported by other authors, as mentioned below. Thus, the NPN content determined using micro-Kjeldahl method, ranged from 490 to 610 mg/100 g for field bean (Drumm *et al.*, 1990), from 160 to 730 mg/100 g for chick pea (Singh *et al.*, 1981*b*; Singh & Jambunathan, 1982), and is considered 390 mg/100 g for lentil (Holt & Sosulski, 1981) and 700 mg/100 g for faba bean (Khalil & Mansour, 1995). However, in the present study, the

Table 3. Protein nitrogen solubilized by the three extraction methods used to obtain the NPN content of six Spanish grain legumes (mg/100 g on a dry weight basis)[a]

Legume	Extraction method		
	EtOH	EtOH + TCA	NaOH + TCA
Chick pea	0.10 ± 0.03	2.21 ± 0.14	0.15 ± 0.01
Faba bean	0.64 ± 0.07	2.53 ± 0.04	0.25 ± 0.06
Field bean	0.04 ± 0.01	1.14 ± 0.02	0.12 ± 0.03
Lentil	0.11 ± 0.04	2.29 ± 0.36	0.37 ± 0.09
Lupin	0.04 ± 0.01	0.41 ± 0.03	0.24 ± 0.05
Pea	0.58 ± 0.05	1.05 ± 0.09	0.73 ± 0.11

[a]Results are mean values ± SD of three replicates assayed by Lowry's procedure.

NPN content of pea was higher than the values reported by other authors, such as Bhatty *et al.* (1973), who found values ranging from 129.5 to 662 mg of NPN/100 g of the dry weight of pea seeds, depending on the cultivar and extraction method, and Chen & Thacker (1978), who reported NPN values between 263 and 337 mg/100 g of dry matter in three different cultivars of green peas.

Bhatty *et al.* (1973) considered that the EtOH method gave the most accurate picture of the NPN content, because of the poor solubility of oilseed and pea reserve protein in ethanol. The same authors suggested that when a high TCA concentration (of around 30%) is used to solubilize the NPN in oilseeds and peas, there is a partial hydrolysis of the protein, leading an overestimation of the seeds' NPN content. On the other hand, Singh & Jambunathan (1981) showed that extraction of meal N using a TCA concentration of up to 10% did not cause protein hydrolysis and even brought about a decrease in the solubility of the meal nitrogen.

In Table 3 we show the protein N solubilized by the three extraction methods used to solubilize NPN, as determined by Lowry's method. With the EtOH and NaOH + TCA methods a little protein N was solubilized, whereas with EtOH + TCA a considerable amount of protein N was solubilized in chick pea (2.21 mg/100 g), faba bean (2.53 mg/100 g) and lentil (2.94 mg/100 g). The high concentrations of TCA (30%) used in the NaOH + TCA extraction method did not result in an increase in protein N solubilization. Singh & Jambunathan (1981) reported that low TCA concentrations (less than 10%) solubilized higher quantities of protein N. In general, to avoid this effect and according to the data obtained in the present study, a protein N assay is required to ascertain the true value of the NPN content being evaluated.

Tables 4–6 give the free amino acid composition of the NPN fractions in the legume seeds, solubilized with each extraction method. The legumes contained all the protein amino acids in the NPN fraction, but the profile varied depending on the legume and extraction method. In general, the common amino acids detected by the three methods were aspartic and glutamic acids, whereas cystine and methionine were present in lower concentrations, which did not exceed 0.76 and 1.44 mg g^{-1}, respectively. This pattern is to be expected, considering that the amino acid profiles of chick pea (Singh *et al.*, 1981; Singh & Jambunathan, 1982), faba bean (Khalil & Mansour, 1995), field bean (Wu *et al.*, 1994), lentil (Savage, 1988) and pea (Savage & Deo, 1989) are characterized by a low concentration of methionine and cystine and a high concentration of glutamic acid, aspartic acid and lysine. However, lysine as well as the other basic amino acids, histidine and arginine, were found in the NPN fraction in lower concentrations than glutamic and aspartic acids. The lower extraction values obtained for basic amino acids with EtOH and EtOH + TCA as compared to NaOH + TCA, may be due to their low solubility in ethanol (Bhatty *et al.*, 1973). The NPN fractions, particularly those solubilized in EtOH + TCA and NaOH + TCA, contained high concentrations of ammonia, which was probably mostly derived from amide N hydrolysis during the extraction

Table 4. Free amino acid composition of the non-protein nitrogen fractions of some legumes extracted with 70% ethanol (mg g^{-1} on a dry weight basis)[a]

Amino acids	Chick pea	Faba bean	Field bean	Lentil	Lupin	Pea
P-Ser	—	0.05	—	0.48	—	—
Asp	0.19	0.46	0.93	1.85	0.06	0.17
Thr	0.13	0.07	0.80	0.48	0.75	—
Ser	0.08	—	0.81	0.38	0.25	0.07
Glu	0.37	0.36	3.19	2.54	0.55	—
Pro	—	—	3.11	3.17	0.19	—
Gly	0.03	0.06	0.37	0.47	0.19	—
Ala	0.11	0.12	1.10	0.42	0.81	0.01
Val	—	—	1.06	0.19	0.43	—
Cys	0.31	0.21	—	—	—	—
Met	—	—	—	—	—	—
Ile	—	—	1.66	—	0.32	—
Leu	—	—	—	—	0.45	—
Tyr	—	—	1.55	0.59	0.08	—
Phe	0.19	—	—	0.92	0.18	—
GABA	—	—	2.43	0.15	0.45	—
Lys	—	—	1.35	—	0.21	—
His	—	2.21	—	—	—	0.036
Arg	—	—	2.51	—	0.59	—
Ammonia	—	—	4.90	—	0.43	—

[a]Results are mean values of three replicates.

Table 5. Free amino acid composition of the non-protein nitrogen fractions of some legumes extracted with 70% ethanol + 1% TCA (mg g^{-1} on a dry weight basis)[a]

Amino acids	Chick pea	Faba bean	Field bean	Lentil	Lupin	Pea
P-Ser	—	—	0.83	0.92	—	—
Asp	0.17	0.16	0.45	0.99	0.14	—
Thr	0.15	0.05	—	0.45	0.01	—
Ser	—	0.04	—	0.69	0.38	0.24
Glu	0.67	0.94	1.10	1.67	0.63	0.94
Pro	0.19		—	3.02	0.66	—
Gly	0.01	0.09	0.02	0.48	0.34	—
Ala	0.34	0.13	0.43	0.84	1.15	0.15
Val	—	0.21	0.19	1.70	0.76	0.02
Cys	—	0.34	0.26	0.35	0.11	—
Met	—	0.12	—	0.17	0.12	—
Ile	0.12	—	0.14	1.62	0.63	—
Leu	—	0.18	—	3.09	0.78	—
Tyr	0.05	0.10	0.10	—	0.33	—
Phe	—	0.17	0.10	1.63	0.48	—
GABA	0.04	0.08	0.09	1.53	0.65	—
Lys	—	—	—	—	0.42	—
His	—	—	—	1.05	0.14	—
Arg	—	1.57	—	2.24	0.80	—
Ammonia	4.85	1.79	2.33	2.60	0.59	1.42

[a]Results are mean values of three replicates.

Table 6. Free amino acid composition of the non-protein nitrogen fractions of some legumes extracted with 0.2% NaOH + 30% TCA (mg g^{-1} on a dry weight basis)[a]

Amino acids	Chick pea	Faba bean	Field bean	Lentil	Lupin	Pea
P-Ser	1.64	0.72	1.14	—	0.92	0.47
Asp	—	—	0.31	1.32	—	—
Thr	0.06	—	0.28	0.28	—	0.10
Ser	0.27	0.90	0.53	0.22	0.58	3.58
Glu	5.49	6.43	6.03	—	—	1.73
Pro	—	—	2.13	—	1.15	2.84
Gly	0.74	0.50	0.55	—	—	4.01
Ala	0.65	0.29	0.61	0.53	0.83	0.96
Val	0.31	—	—	—	—	0.47
Cys	0.24	0.24	—	0.60	—	0.76
Met	0.36	—	1.44	0.54	—	0.31
Ile	0.64	—	—	—	—	3.48
Leu	0.38	—	—	—	—	4.91
Tyr	0.18	0.04	—	0.87	—	1.33
Phe	0.32	0.11	1.21	0.32	—	2.84
GABA	0.04	0.11	0.26	—	—	5.31
Lys	0.63	0.19	—	—	—	4.35
His	0.91	0.16	0.36	0.33	1.05	0.98
Arg	5.65	—	—	0.19	—	7.38
Ammonia	2.46	2.92	0.04	0.33	—	5.30

[a]Results are mean values of three replicates.

process. This high ammonia contents detected in all legumes except lupin may also be due to the presence of some free ammonia in these seeds, as has been reported for pea seed (Bhatty *et al.*, 1973) and for red kidney bean (Wu *et al.*, 1994).

CONCLUSIONS

When the values of TN–PN (Table 1) are compared with the NPN content which is solubilized using the three extraction methods (Table 2), there is a marked difference in the values obtained, which might be due to the solubility of amino acids in the extraction solvents used and to the solubility of other nitrogen compounds made up of nitrate nitrogen and nucleic acid nitrogen (Holt & Sosulski, 1981). For this reason, it is difficult to ascertain or recommend an appropriate extraction method for obtaining the true NPN content of legumes since the results vary widely and none of three extraction methods tested was totally accurate for determining the NPN content of grain legumes. However, the estimating value of NPN as TN–PN could be considered as an useful method for labelling purposes, but further studies should be made in an attempt to obtain a suitable extraction method for measuring total NPN in legumes.

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